Minicells, back in fashion: Bacterial minicell is a powerful toolbox for *in situ* structural biology

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Introduction

Cryo-electron tomography (cryo-ET) of bacteria is quickly emerging as a powerful tool for the *in situ* visualization and 3D reconstruction of macromolecular structures within intact cells. Recent advances in sample preparation and image processing techniques are faithfully producing images of structures at nanometer scale resolution. Historically, traditional electron microscopy (EM) was limited to producing images of subcellular structures that lacked molecular resolution. Available methods for preparing samples for EM analysis such as sample fixation perturbed the native state of cells, causing artifacts and limited image information. In 1983 Dubochet et al. reported the development of a technique for preparing samples for EM analysis (vitrification) where biological samples are quickly frozen in a way that preserves the native structure of cells. One of the physical limitations of EM is the inability to generate useful information from samples greater than 500 nm thick. Thin sectioning of samples prepared by conventional methods and by vitrification is one way of overcoming this limitation but is also prone to artifacts. This limitation has restricted use of cryo-ET to bacterial species that are less than 500 nm thick or to imaging thinner regions of larger cells, such as polar regions (1-3). Much has been learned from analyzing various macromolecular structures in bacterial species conductive to Cryo-ET: Analysis of the cell division septum in *Caulobacter crescentus* unveiled that the cell division protein FtsZ forms short, arch shaped filaments around the division septum instead of a continuous ring as once proposed (4). The first high-resolution structures of flagellar motors were determined in *Treponema primitia* and *Borrelia burgorferi* (5). The organization of the chemoreceptor signaling process was first determined in *C. crescentus* cells (6). For a through review of successful application of cryo-ET in whole cell bacteria please refer to the following review (7). A novel strategy that overcomes cell thickness limitations for in situ imaging of macromolecular structures that has produced high-resolution 3D density maps employs the use of bacterial minicells.

Minicells, nano-sized (1 – 0.5 uM), a-nucleated particles produced by abnormal cell division that occurs near a cell pole were first described in 1966 by Adler et al. in a mutant strain of *Escherichia coli* and also later described in other species of bacteria (8-11) (Fig. 1A). A multitude of studies characterizing their general properties found that minicells remain metabolically active for a finite amount of time and retain many of the biochemical and structural properties of the normal sized parental cell. Minicells lack chromosomal DNA and thus cannot divide but can continue other cellular processes such as ATP synthesis, replication and transcription of plasmid DNA and translation of RNA. It was also found that plasmid-carrying minicells can act as genetic donors during conjugation and that phage are capable of infecting minicells. These unique properties led many to exploit minicells as *in vitro* systems for the study of cellular processes in the absence of chromosomal DNA(12-14). Genetic and biochemical characterization of *E. coli* minicell producing mutants revealed that the genetic deficiencies in these strains code for a unique system known as the Min system that mediates the proper mid-cell placement of the cell division septum(15). In recent years, minicell use has been proposed in the development of

vaccines, and “target drug delivery systems” where minicells are engineered to deliver cytotoxic chemotherapeutics specifically to cancer cells(10, 16). Thus far minicells have been used for developing high-resolution structural models of the infection process of various types of bacteriophage, bacterial chemoreceptors and type III secretion systems (T3SS)(17-24). Considering the fact that bacterial secretion systems are known to be involved in many pathogenic processes and in promoting the development of antimicrobial resistance, the continued use of minicells for the structural elucidation of secretion systems from various bacterial pathogens will prove useful for understanding more about the molecular machines that bacteria use to make people sick. Here we will review the various methods for producing, isolating and purifying minicells as well as review successful examples of their use in cryo-ET.

The Min System

The Min system is best characterized in *E. coli* and *Bacillus subtilis* and is conserved among a majority of bacterial species. In E. coli this system is composed of three proteins, MinC, MinD, and MinE that synergistically mediate the proper placement of the cell division machinery, or the divisome, by inhibiting its development at sites other than mid-cell. The divisome is a multi-protein complex that mediates the reconstruction of the cell envelope to form two daughter cells from a single cell during the cell division process. FtsZ, a bacterial homolog of tubulin is an essential, pioneering component of this complex that initiates the development of the divisome by forming a ring-like structure, termed the Z-ring, at a potential site of division and recruits additional divisome protein constituents. The Min system mediates the proper placement of the divisome through negative interactions with FtsZ that inhibit Z-ring formation. The primary FtsZ antagonist of this system, MinC, is known to interact directly with FtsZ, preventing it’s polymerization into a ring. MinD and MinE interact with MinC in a way that restrict MinC’s cellular localization to polar zones thus reducing mid-cell MinC concentrations to levels conductive for Z-ring formation. Thus, mutant strains of *E. coli* deficient in Min system function are no longer able to exclusively localize the Z-ring to mid-cell and are able to develop a polarly localized division septum who’s division product is a minicell, along with a normally localized septum at mid-cell that generates viable daughter cells(25).

Generating minicell producing mutants

Inactivation of the Min system has proven successful in generating minicells from many species including *Salmonella enteric*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Corynebacterium glutamicum*, and *Bacillus subtilis* (26). Inactivation of the Min system in some bacterial species may not be sufficient for generating high minicell yields or minicells that are of appropriate size for cryo-et analysis. For example, *Listeria monocytogenes* Min system inactivation produces minicells at very low frequency. One approach for increasing minicell yield has been successfully implemented. *For L. monocytogenes*, FtsZ over expression from a plasmid in a Min deficient strain lead to a two fold increase in minicell production (27). In fact FtsZ overexpression alone in a wild type strain of *E. coli* was sufficient for producing minicells (28). Overexpression of FtsZ

may also be an appropriate strategy for generating minicells in bacterial species where the mechanism that mediates divisome placement is not known.

Alternative mechanisms for mediating divisome placement have been identified in a few species of bacteria (29). One example is found in *Myxococcus xanthus* where a novel protein called PomZ positively regulates assembly of the divisome by recruiting FtsZ to mid-cell, cells deficient for PomZ readily produced minicells (30). Inactivation of these systems should be considered when attempting to generate minicells from species lacking the Min system.

Tiny Minicells

Generating a novel mutant strain of bacteria capable of producing minicells can be relatively straight forward since the genetic elements that code for Min systems are generally well conserved in most bacterial species. Generating minicells that conform to the size requirements conductive for Cryo-ET may be more complicated since minicells are usually 1/10th the thickness of the parental strain. In *E. coli* simple inactivation of the Min system generates minicells that are about 1 uM thick. Several strategies for generating mutant strains of bacteria capable of producing minicell that conform to the dimensional parameters conductive for cryo-ET have been employed.

The first minicell system specifically designed for use in cryo-et analysis (developed to study bacteriophage-host interactions) generated tiny minicells from a strain of E. coli with a mutation in the cell-shape deterring protein MreB (Fig.1B). This strain had previously been shown to produce a cell shape defect that generates cells with a thinner diameter than wild-type cells. The Min system in this skinny cell *E. coli* strain was inactivated by deleting the Min operon and resulted in minicells with a reduced diameter that was adequate for cryo-et analysis (17). An alternative approach for obtaining minicells of smaller diameters is to use a multistep minicell isolation technique to isolate minicells of desired diameter.

Isolating minicells

Various methods for isolating minicells have been employed and selection of a particular process depends on application. When isolating minicells from cell systems designed to produce tiny minicell, differential centrifugation is perhaps the simplest method for minicell enrichment, see the following (12) for details. A more complex isolation method designed to isolate minicells of uniform size and free of parental cells (10) is recommended when preparing minicell from systems not optimized for generating tiny minicells. In this approach cultures of minicell producing strains (Δ minCDE) are grown under high-stress conditions (5% NaCl) known to cause bacterial cells to become filamentous. Next, bacterial cultures are centrifuged at 2,000 g for 10 min to remove the majority of large cells. Supernatants are collected and minicells are enriched by three sequential density gradient centrifugation steps. Enriched minicell suspensions are then passed through a 0.45 um cross-flow filter to homogenize

minicell size. This method was reported to produce high minicell yield (10^{10} from 6L of starting culture) with a uniform size of about 400 nm.

Bacteriophage - Host Interactions

Developing a greater understanding of the structural properties and conformational dynamics associated with bacteriophage (phage) – host interaction is an area of research that will benefit greatly by the use of minicell systems for cryo-ET analysis. Phage are the most abundant biological entity on earth, only a small fraction of their diversity is known and much less is understood about the various molecular strategies employed by phage to infect their host. Phage infection plays a significant role in influencing microbial diversity and promoting the evolution of bacterial genomes in ways that can influence human disease(31, 32). In multi-species bacterial communities associated with the GI tract of humans, phage can play a role in pathogenesis by reducing bacterial diversity in response to adverse environmental stress (antibiotic treatment) that cause pro-phage to enter their lytic cycle(33). The resulting condition called dysbiosis, is characterized by the enrichment of potentially pathogenic bacteria, promoting disease of the GI tract. Phage genomes can become incorporated into the bacterial chromosome, promoting the acquisition of new genes and thus bacterial evolution. For example, the human pathogen *S. enterica* encodes a homolog of a phage muramidase gene involved in the secretion of the typhoid toxin. The recently described type VI secretion system found in Gram-negative bacteria is structurally and mechanistically analogous to a contractile phage tail (34). These phage-like bacterial systems were likely incorporated into bacterial genomes by phage interactions. In light of the rapid rise of resistance to clinical available antibiotic, phage also hold much potential in the development of novel anti-bacterial treatments. The importance of phage in understanding and manipulating human interactions with the bacterial world warrants intensive studies of phage-host interactions.

Three basic steps characterize the phage infection process: initial attachment of a phage particle to the surface of a bacterial cell, penetration of the cell envelope, and injection of the phage genome into the host. Phage attach to bacterial cell surfaces via interactions between phage tail proteins and receptors proteins or LPS on the bacterial surface. The type of bacterial receoptoers that associate with phage are diverse and include flaggerla proteins and proteins associagted with type IV pili. After attachment the phage particle undergoes conformational changes that mediate penetration through the cell envelope and the translocation of genetic material into the host. Although multiple studies have been able to characterize biochemical, and structural properties of isolated phage particles, the visualization of phage-host interactions has remained more elusive. The first cryo-ET characterization of interactions between a phage particle and a membrane structures (proteoliposome) were first reported by Bohm et al. in 2001 and revealed conformational changes in T5 phage that mediated the passage of the phage tail through the lipid bilayer. Proteoliposomes were used in this study to overcome the limited image information that would result by using whole cells in the cryo-et process.

The first in situ characterization of phage – host interactions came after the development of the tiny minicell system of E. coli, specifically developed for use in cryo-ET analysis. In this study, the three stages of P1 phage infection of the minicell host was captured and 3D models were developed (17). The P1 phage has icosahedral head that holds the phage genome, a contractile tail that is composed of two primary structures, the tail tube and the tail sheath (a structure surrounding the tail) and six tail fibers involved in targeting the cell surface receptor (LPS in this case). P1 phage were observed at three distinct stages of infection. After initial attachment it was found that the base of the contractile tail sits about 53 nm over the cell surface and is about 225 nm in length. In the next stage of infection the tail sheath contracts towards the head, is reduced in length to about 105 nm and the tail tube projects into the cell membrane. After tail contraction, an image density once observed in the phage head disappears, indicating the translocation of the phage genome into the minicell.

A subsequent study by the same group pushed the limits of resolution and provided a example of the extraordinary potential minicell systems have for cryo-ET studies (18). The phage infection process between the T7 bacteriophage and a tini minicell E. coli host was characterized to a 4 nm scale resolution. The T7 bacteriophage has a short non-contractile tail, an icosahedral capsid structure, a internal protein core and six tail fibers. The tail fibers of a non-host associated T7 phage were found pointing towards and tighly packed around the head structure (previous EM analysis do not describe tail fibers). A transient association of extended tail fibers pointing down towards, and making contact to the cell surface was observed. The weak association between tail fibers and their cognate cell surface receptors is postulated to represent a strategy that increases the chance for phage particles to infect new cells; high affinity between tail fibers and cell surface receptors would increase the chance of multiple phage particles infecting a single cell. It is also proposed that it is not until after the phage tail makes contact with it's cognate receptor that all tail fibers become associated to the cell surface. An intermediate conformation was observed where the tail made contact with the membrane but did not protrude through the cell envelope. After the phage was stably associated with its host, the elaboration of a tail structure originating from the phage capsid was observed followed by injection of genome DNA into the cells (Fig. 2). One additional example of phage – host interactions have been studies using a minicell system, please refer to the following publication (19).

Bacterial chemotaxis is a process by which bacteria are able to sense the chemical composition of their environment and respond to changes by either moving away from unfavorable or towards favorable conditions. This process is mediated by a polarly localized, membrane bound complex of proteins (chemotaxis signaling complex) that sense chemical signals and transduces the signal to motility organelles to modulate the direction of bacterial movement. The chemotaxis signaling complex is composed of three protein constituents: methyl-accepting chemotaxis protein (MCP), CheA (histidine

kinase) and CheW (complex coupling protein). Analysis by various biochemical techniques have determined crystal structures of individual components and revealed inter-protein interactions(35). The spatial organization of these proteins into a functional complex was analyzed by cryo-ET imaging of whole *Caulobacter crescentus* cells(6). These studies produced low-resolution structures of the chemotaxis signaling complex revealing that MCPs are anchored into the cytoplasmic side of the inner-membrane forming pillar like structures and that CheA/W interact with the cytoplasmic tip of MCPs forming a baseplate structure. It was also found that MCPs form functional units that consist of trimers of MCP dimers that form triangular structures when viewed from an angle parallel to the membrane and that functional units are further organized into hexagonal arrays that consist of 6 MCP functional units that are linked together at the cytoplasmic end by the CheA/W base plate.

Cryo-ET studies of the chemotaxis signaling complex in minicells produced from a *Salmonella enterica* strain engineered to overexpress FtsZ generated higher resolution 3D tomograms where individual MCP dimers where clearly visible(20). A known crystal structure of an MCP dimer complex was docked into the 3D map generated by this study confirming that the quaternary crystal structure of the MCP dimer is physiologically relevant. It was also found that individual pillars of receptor dimers appear straighter near the baseplate than what was observed in crystal structures and that a bend is seen near a glycine residue known to be important for receptor function (Fig. 3 A-C). Although the resolution obtained in this study was sufficient to accurately model the 3D structure of the MCP component of the signaling complex, it was not sufficient to elucidate the structural organization of the CheA/W base plate. Higher resolution tomographs obtained by imaging chemoreceptor arrays in tiny E. coli minicells revealed sufficient detail to generate a density model of a complete chemotaxis-signaling complex and revealed the molecular basis of receptor array formation(21). It was observed that the triangular structures formed by MCP functional units form hexagonal arrays where a tip of the triangular structure are oriented towards a central point instead of pointing away from each other as described by other models. Density maps also revealed that two adjacent MCP trimers are connected by a continuous density layer. Atomic structures of individual complex components were docked into the 3D density map revealing that the cytoplasmic tips of two MCP trimers are associated with CheW and the P3 and P5 domains of CheA. It was also found that CheW forms hexameric rings that associate with six MCP trimers and that the P3 and P5 domains of CheA along with CheW are involved in interconnecting hexagonal units into an array (Fig. 3 D-F).

Type III Secretion Systems

T3SSs are unique types of molecular machines found in many Gram-negative bacteria that mediate the secretion of proteins from the cytoplasm into, and across the cell envelope. There are two types of T3SS families each involved in elaborating unique molecular machines, the flagellar T3SS that mediates swimming motility and the non-flagellar T3SS (the injectosome) a system involved in translocating and injecting virulence factors into eukaryotic host cells. These two systems diverge greatly in terms

of form and function but share a common, evolutionarily related core structure which consists of a multi-ring basal structure that transverses the periplasmic space and is embedded into the inner and outer membranes, a basal body which consists of an export apparatus, a sorting platform and an ATPase that connects to the multi-ring basal structure at its cytosolic end. Injectosomes are simpler machines compared to flagellar systems and are composed of approximately 20 protein constituents that together form a macromolecular machine that mediate the secretion and assembly of a needle-like appendage that transverses and protrudes from the bacterial cell envelope, makes direct contact with a eukaryotic host cell and serves as a conduit for the delivery of bacterial effector proteins directly into host cells. Flagellar T3SSs are much more complex, consisting of approximately 40 protein constituents that mediate the secretion and assembly of the flagellum and a rotary motor which connects to the flagellum, providing the force needed to drive rotation(36-39).

Flagellar T3SSs

There are two types of flagellum-mediated motility described in bacteria. The first and most common is characterized by an extracellular flagella where rotation of the flagella propels a cell through its environment. The second type of flagella mediated motility is rare and unusual, found only in Spirochetes and is characterized by the elaboration of inner-membrane anchored flagellar motors at polar zones of the cell and the compartmentalization of the flagella into the periplasmic space. The periplasmic rotation of flagella produce cork-screw like cell morphology and cause a serpentine-like movement of the cell body, allowing cells to burrow through viscos mediums. Most of the structural information known about flagellar T3SSs is derived from biochemical and structural analysis of flagellar motors isolated from bacteria. The first 3D model of an in situ flagellar motor was first developed by cryo-ET analysis of the spirochete periplasmic flagellar system. This system was chosen for analysis due to the small diameter of cells from this phyla but its structure is not representative of flagellar systems where the flagellum protrudes out of a cell(5). The lack of an in situ 3D structural model of the extracellular flagellar T3SS represents an excellent opportunity for the development and employment of minicell systems for acquiring high-resolution tomograms using cryo-ET.

One study focusing on a comparison analysis of conserved structures between the flagellar and injectosome T3SSs employing the use of minicells derived from *Salmonella enterica* did not aim at generating an in situ model but this study represents the potential of minicell systems for generating a high-resolution model of the flagellar T3SS(22). Genetic, biochemical and phylogenetic analysis of T3SSs have indicated that flagellar and non-flagellar core system components are evolutionarily related but their structural similarity in situ was not known. *S. enterica* is a Gram-negative pathogen of the gastrointestinal (GI) track and posses both flagellar and non-flagellar T3SSs. A minicell producing mutant was generated by overexpressing FtsZ. The minicell strain was found to produce flagellated minicells that were active in motility in liquid medium and had an average thicnkness of 500 nm. Minicells were prepared for cryo-et analysis by vitrification. Tomograms of these *S. enterica* cells revealed that minicell bodies

contained both flagellar and injectosome T3SS which allowed for direct comparison of distinct machines embedded in a common cell envelope. The most commonly conserve protein constituents found in both types of T3SSs compose the inner membrane-embedded export gate and a cytoplasmic complex named the sorting platform that includes an ATPase. Density maps revealed previously uncharacterized structures in the injectosome T3SS that had structural similarities to previously characterized structures of the flagellar export gate and sorting platform (Fig. 4, A-D).

Injectosome T3SSs are essential virulence factors that contribute to the pathogenesis of infectious disease caused by many Gram-negative bacterial pathogens. T3SS are associated with bacteria-host interactions and are exclusively involved in promoting virulence in pathogenic species. These systems mediate the secretion of virulence factors, commonly referred to as effector proteins that are diverse in nature and have profound effects, modulating normal host cell function in a way that promotes adherence, invasion and persistent colonization of a host (40). Although the general genetic and biochemical characteristics of T3SSs are generally well conserved among bacteria, recent phylogenetic analysis has discovered that these systems can be categorized into specific clades based on their evolutionary origin and fall into groups associated with host interactions(38). The diversification of injectosomes is thought to have occurred not through the evolution of effector proteins with host specificity or through the diversification of commonly conserved units of T3S but structural components involved in producing the needle and the tip complex. Diversification of the needle and tip complex was likely due to selective pressures associated with distinct membrane characteristics of the host cell. In light of the dramatic rise of resistance to almost all clinically used antibiotics among bacterial pathogens, T3SSs are emerging as promising targets for the development of novel antibacterial drugs that block virulence by preventing the export of effector proteins into a host cell. Since the structural elements of T3SSs associated with making direct contact with host cells from different clades are perhaps structurally different, developing high-resolution molecular structures of T3SS of different groups will be important for designing inhibitors that block injectosome function.

Minicells systems have been employed in two studies characterizing the structural organization of the injectosome through cryo-ET. One study analyzing the injectosome from the GI pathogen *Yersinia enterocolitica* was able to produce an in situ model of the injectosome multi-ring basal structure, the needle complex and the export gate but did not achieve sufficient resolution to image the protein sorting complex(23) (Fig. 5, A-C). A subsequent study focused on characterizing the injectosome of *Shigella flexneri* developed a model system that generated minicells that were 300 nm thick and was conductive for the genetic manipulation of injectosome components for elucidating how the absence of specific proteins affected the structure(24). Minicell producing strains were generated by introducing a plasmid that constituitivley expresses E. coli versions of ftsQ, ftsA, and ftsZ (genes known to cause minicell formation when over expressed) into Wild-type and injectosome mutant strains of *S. flexneri*. In order to determine if injectosomes expressed in minicells were functional, purified minicells

containing WT versions of injectosomes were tested for hemolysis activity. Hemolysis in *S. Flexneri* is known to be mediated by the secretion of effector proteins into blood cells via a injectosome mediated pathway. It was found that minicells induced hemolysis. CryoET imaging was performed of minicells making direct contact with red blood cells via an injectosome appendage. These studies confirmed that minicell injectosomes are able to translocate proteins into eukaryotic cells. 3D density maps from cryoET analysis revealed a molecular resolution of 2.7 nm and the previously uncharacterized structure of the sorting platform. Minicells from mutant strains containing single deletions (*ΔmixN* or *Δspa33*) in genes postulated to code for proteins that make up the sorting platform in flagellar T3SS (based on homology) were analyzed. MxiN and Spa33 are known to be required for secretion and assembly of the needle complex but not for assembly of the basal body. Tomograms of both mutant strain revealed that the needle complex was absent but that basal body was present, confirming that MxiN and Spa33 are required for needle complex assembly (Fig. 5, D-I).

Future Perspective

The development of minicell systems has great potential for applications that combine in situ structural visualization of macromolecular structures combined with genetic and biochemical analysis. The early days of minicell research found that high copy plasmids can segregate into the minicell and mediate expression of plasmid genes. This one characteristic of tiny-minicell systems has great potential for elucidating the necessity and sufficiency of genetic elements postulated to code for proteins that compose specific macromolecular machines. For example in studies analyzing the structure of T3S systems, tomograms indicate that there are densities within 3D maps where the identity of a corresponding protein is unknown. Generating tiny-minicell expression systems where all known structural proteins are accounted for, will help resolve the issue of these orphan densities.

In situ models of other secretion systems such as type II secretion associated type IV pili will help us understand how these organelles mediate bacterial motility, attachment and how these type of systems ultimately contribute towards pathogenesis of disease in man. Interactions between bacteriophage and notable infection related receptors on the bacterial cell surface (such as type IV pili or flagellar proteins) will help in understanding how phage play a role in the transition between the planktonic and biofilm lifestyle of bacterial species (*Psudomonas aeruginosa*) known to depend on phage lifecycles for their own development. Incorporating genetic, biochemical, and cryo-ET analysis of these type of analysis will help elucidate how specific protein domains and individual residues contribute to the overall structure and function of these molecular machines in bacteria. The End!

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